REMARKS/ARGUMENTS

Claims 1, 5-7, 12-17 and 19 are now pending in this application.

Claim 12 has been amended to correct a minor typographical error. No new matter has been added.

Rejections under 35 U.S.C. §102 and §103

The only outstanding rejections were made over PCT publication WO 99/15652 to Miyata et al.

The Examiner has pointed out that the rejection can be overcome by filing a certified translation of the priority document thereby establishing that applicants had possession of the invention as of March 19, 1999. Applicants have attached hereto a certified copy of the priority document thereby overcoming the rejection.

Applicants appreciate the Examiner's indication that the earlier rejections have been withdrawn. In view of such and in view of the attached certified translation the application is believed to be in condition for allowance and an indication of such is respectfully requested.

The Examiner will note that the attached certified translation includes a verification statement indicating that the translator is well acquainted with both English and Japanese languages and that the attached document is a true and correct translation.

Applicants point out that Examples 7 and 8 on pages 34 through 36 of the attached translation disclose a diagnostic method. The diagnostic method is a method of diagnosing mesangial cell proliferative neuropathy. Such a method is claimed within claims such as claims 1 and 5 now pending in the application.

The examples 7-8 on pages 34-35 also disclose the reagent as well as the sequences such as those referred to within claim 6. The Examples 7-8 on pages 34-35 of the application are the working Examples 9 and 10 on pages 35-37 of the present application.

The method as claimed in claim 1 and the reagent as claimed in claim 6 which includes the specific SEQ IDs is encompassed by independent claim 12 which is supported at the same places in the specification.

Lastly the Examiner will note that kits useful in such methods as claimed within claim 17 are referred to in the translation such as in the Abstract on page 65 at lines 8-10.

In view of the attached verified translation of the priority document it is applicants position that the application is in condition for allowance and an indication of such is respectfully requested.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number SHIM-012.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: _5/May/05_
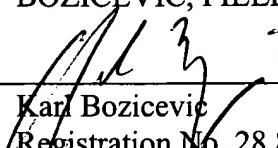
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Attachment: Certified Translation

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VERIFICATION OF TRANSLATION

I, <u>Mariko Ogura</u>

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declare as follows:

1.   That I am well acquainted with both the English and <u>Japanese</u> languages, and

2.   That the attached document is a true and correct translation made by me to the best of my knowledge and belief of:

(a)   Japanese Patent Application No. Hei 11-075305
      Entitled: "A METHOD FOR DETECTING MEGSIN PROTEIN AND USE
            THEREOF"
      Filed on March 19, 1999

April 26, 2005
(Date)

Mariko Ogura

(Signature of Translator)
Mariko Ogura

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    【Registration Number】 041092

    【Amount】 21000

【List of Attached Documents】

    【Name of Document】 Description   1

    【Name of Document】 Drawings   1

    【Name of Document】 Abstract   1

【Proof】 Necessary

[Document Name] Specification

[Title of the Invention] A METHOD FOR DETECTING MEGSIN PROTEIN AND USE THEREOF

[Claims]

[Claim 1]　A method for evaluating renal functions, which comprises measuring the megsin protein in a biological specimen.

[Claim 2]　The method for evaluating renal functions of claim 1, which comprises measuring the megsin protein in a biological specimen, and comparing it with the megsin protein amount in a normal specimen.

[Claim 3]　The method for evaluating renal functions of claim 1, wherein the biological specimen is urine.

[Claim 4]　The method for evaluating renal functions of claim 1, wherein the megsin protein in the biological specimen is measured by an antigen-antibody reaction using an anti-megsin protein antibody.

[Claim 5]　The method for evaluating renal functions of claim 4, wherein the anti-megsin protein antibody is a monoclonal antibody.

[Claim 6]　A reagent for diagnosing renal functions, which comprises the anti-megsin protein antibody.

[Claim 7]　The reagent for diagnosing renal functions of claim 6, wherein the anti-megsin protein antibody is a monoclonal antibody.

[Claim 8]　A granule for detecting megsin protein in a biological specimen, wherein the granule comprises a solid granule to the surface of which an anti-megsin protein antibody is bound.

[Claim 9]　The granule for detecting megsin protein of claim 8, wherein the solid granule is magnetic.

[Claim 10]　The granule for detecting megsin protein of claim 8, wherein the relative density of the solid granule is not smaller than 1.

[Claim 11]　The granule for detecting megsin protein of claim 8, wherein the anti-megsin protein antibody is a monoclonal antibody.

[Claim 12]　A method for detecting megsin protein in a biological specimen, comprising the following steps of:

i) contacting the granule for detecting megsin protein with the biological specimen, said granule comprising a solid granule to the

surface of which an anti-megsin protein antibody is bound;

ii) contacting the second anti-megsin protein antibody bound to a marker molecule with said granule for detecting megsin protein to which the biological specimen was contacted; and

iii) detecting the marker molecule bound to the megsin protein through the second anti-megsin protein antibody.

[Claim 13] The method for detection of claim 12, wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are both monoclonal antibodies.

[Claim 14] The method for detection of claim 13, wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are antibodies having different recognition sites.

[Claim 15] The method for detection of claim 12, wherein the biological specimen is urine.

[Claim 16] The method for detection of claim 12, wherein the biological specimen is blood.

[Claim 17] A kit for detecting megsin proteins, which comprises the following elements:

a) magnetic solid granules to which anti-megsin protein antibodies can be bound,

b) anti-megsin protein antibodies, which are bound to said magnetic solid granules in advance, or can be bound to them indirectly, and

c) a magnet.

[Claim 18] The kit for detecting megsin proteins of claim 17, further comprising an anti-megsin protein antibody to which a marker molecule is bound.

[Detailed Description of the Invention]

[0001]

[Technical Field of Industrial Application]

The present invention relates to a method for evaluating renal function, which comprises measuring the megsin protein in biological specimens such as urine, blood, and so on. Furthermore, the present invention relates to reagents for diagnosing renal function comprising anti-megsin protein antibodies.

[0002]

[Prior Art]

Kidney transplantation is the only method to treat terminal renal failure where the renal functions of hemofiltration and detoxication fail completely. However, the supply system supporting renal transplantations in Japan is far from well structured. In addition, social cognition on the transplantation method itself is not advanced. Therefore, the present status is that there is no alternative but to rely on dialysis as a renal replacement treatment.

Today, the number of patients who undergo dialysis is estimated to be approximately 170,000. An average annual treatment cost of about ¥6 million is needed per patient, and it is considered to be one of the biggest reasons that constrain the health insurance system. Furthermore, treatment with dialysis restraints the patient for 4 to 6 hours a day, 2 to 3 days a week, which greatly hampers the social activities of the patient.

Renal failure is a condition patients with renal diseases finally reach. The cause and background leading to renal failure is diverse. There are many cases in which diseases that usually originate outside the kidneys such as drug poisoning, infectious diseases, malignant tumors, diabetes, systemic lupus erythematosus (SLE), cause renal disorders and finally lead to renal failure.

Significant subjective symptoms of renal disorders appear only at late stages, namely only after the condition has almost worsened to renal failure. Therefore, they are easily overlooked, and numerous cases exist where at the point when symptoms appear, the kidney has already suffered an unrecoverable damage. Thus, it is important to discover renal disorders at an earlier stage as possible before the expression of subjective symptoms in order to prevent transition to renal failure, and also to eliminate constraints dialysis treatment puts on public medical insurance finances.

[0003]

Up to now, the examination of urinary proteins and urinary deposits, the so-called urinalysis, was widely carried out to diagnose renal disorders. However, urinary proteins increase temporarily

even in normal healthy persons due to extreme exercise, psychological stress, abundant meat diet, before menstruation, and so on. On the other hand, there are urinary proteins that are unrelated to renal diseases, such as orthostatic albuminuria found mainly in adolescents (in around 0.5% of normal healthy persons). Urinary proteins are also detected in uropathies, bladder disorders, female genital tract disorders, and so on. Thus, it is difficult to make a definite diagnosis of renal disorders only by examining urinal proteins.

Urine deposits are observed with a microscope after centrifugation of urine. However, erythrocyte deposits are also observed in the urine deposits of normal healthy persons, and may also derive from disorders other than renal disorders, such as those of organs related to the urinary tract. Therefore, urine deposits are also not sufficient enough to make definite diagnoses of renal disorders.

A method to diagnose diabetic nephropathy at an early stage is known, which comprises the following steps: (1) assaying albumin that leaked into the urine as an indicator of diabetic nephropathy; and (2) comparing the value with the normal value of a normal healthy person. However, the exact state of diabetic nephropathy cannot be understood since the albumin content in the urine changes even in a normal healthy person.

In addition, serum creatinine (Cr) and blood urea nitrogen (BUN) are assayed to examine the retention of urinary components in the blood, but this assay is also easily influenced by diet. Thus, abnormal values in assays of urinary proteins, as well as serum Cr and BUN does not necessarily imply a renal disorder, and they may frequently occur in normal healthy persons or patients with other diseases.

[0004]

Further, diagnosis of renal disorders by measuring various substances including urinary β_2-microglobulin, N-acetylglucosaminidase (NAG), IgG, transferrin, interleukin-6, and such are being tried. However, there are many cases where these measurements do not correspond to the severity of the renal disorder, and therefore, are far from being effective.

Moreover, although a disorder of the whole kidney or involvement of immune reactions may be guessed by measuring these blood components in the urine, it is difficult to identify the site within renal tissue that is affected by the disorder. No examination method other than those mentioned above is known for diagnosing renal disorders and determining the severity with a sufficient sensitivity and specificity. Histological diagnosis by renal biopsy is regarded essential for ultimately diagnosing and determining the severity of renal disorders.

[0005]

However, renal biopsy is an invasive examination, and frequently accompanies dangers of complications such as hemorrhage, infections, and so on. Further, to conduct the examination, hospitalization in a well-equipped facility having specialists is required, and the physical and social burden on the patient cannot be ignored.

As mentioned above, the examination by urinalysis is simple and convenient, and is also an excellent examination method that enables treatment of a large amount of specimens. However, from the perspective of providing a definite diagnosis of renal disorders, it is far from being satisfactory. On the other hand, renal biopsy is an authentic method to diagnose and determine the severity of renal disorders. However, its use is highly restricted, which cannot be helped. On this account, a method to diagnose renal disorder, which has the simplicity and convenience of a urinalysis, as well as the accuracy of a renal biopsy, has been desired.

[0006]

Proteins specifically expressed in specific tissues, not only those in the kidney, are often used as indicators of functional disorders of the organ. For example, enzymatic proteins such as LDH and γGTP are widely used as markers of hepatic function. However, no protein specific to the kidneys is known which serves as an indicator of their functions.

The present inventors isolated a gene called megsin as a gene especially strongly expressed in mesangial cells by a macroscale DNA sequence analysis and database analysis, and succeeded in obtaining

the megsin protein comprising 380 amino acids encoded by the megsin full-length cDNA clone. The present inventors, further found that the human megsin protein belongs to the SERPIN (serine protease inhibitor) superfamily (R.Carrell et al., Trends Biochem.Sci., 10:20

5   (1985); R.Carrell et al., Cold Spring Harbor Symp.Quant.Biol., 52:527 (1987); E.K.O.Kruithof et al., Blood, 86:4007 (1995); J.Potempa et al., J.Biol.Chem., 269:15957 (1994); E.Remold-O'Donnell, FEBS Let., 315:105 (1993)) according to an amino acid homology search by the FASTA program using the Swiss Prot database (T.Miyata et al.

10   J.Clin.Invest., 120:828-836 (1998)). These findings were filed as a patent application (PCT/JP98/04269).

Human megsin protein expression was weak in human fibroblasts, smooth muscle cells, endothelial cells, and keratinocytes, and was strong especially in mesangial cells. The comparison of megsin

15   protein expression level in renal tissues from IgA nephropathy patients and normal healthy individuals revealed that the expression level of megsin protein in IgA nephropathy patients was significantly higher. Accordingly, it is possible that megsin protein existing in the urine or blood is useful as a marker of mesangial cell

20   proliferative nephropathy, for example, nephropathies such as IgA nephropathy. There is a possibility that megsin protein gene expression is deeply related with the onset and progression of renal diseases. However, the correlation between megsin protein gene expression and progression of renal disease state was unknown.

25   [0007]

[Problems to Be Solved by the Invention]

An objective of the present invention is to solve problems such as those above, and to provide a method that enables diagnosis of renal diseases and determination of their severity, and also to

30   provide reagents and reagent kits used for the method.

[0008]

[Means to Solve the Problems]

Firstly, the present inventors thought that it is necessary to measure specific proteins related to the state of the disease to make

35   a definite diagnosis of renal disorders and determine their severity, and focused their attention on mesangial cells, which exist in the

glomerulus. The mesangium is mapped on the lobule center in the glomerular tuft and is a tissue that functions as a core to bind the lobules together. The mesangium is covered by the glomerular basement membrane. It is composed of cells (mesangial cells), which

5   are divided from the capillary lumen by endothelial cells, and a three-layered amorphous material (mesangial matrix), which is in sequence with the lamina rara interna in the glomerular basement membrane.

[0009]

10   The mesangial cells play a pivotal role in maintaining the structure and function of the glomerulus. Their proliferation is thought to be a main factor in the onset of glomerular disorders such as glomerular nephritis and glomerulosclerosis. Furthermore, mesangial cells are targets damaged by all types of nephritis. For

15   example, proliferation of mesangial cells and accumulation of extracellular mesangial matrix is thought to be the first step in the development of glomerulosclerosis in patients suffering from various glomerular diseases, such as chronic nephritis and diabetic nephritis, the two main causes of end-stage renal failure(D.

20   Schlondorff, Kidney Int., 49: 1583-1585, (1996); R. B. Sterzel et al., Glomerular mesangial cell. Immunologic Renal Diseases, 595-626 (1997)) Therefore, it is thought that identification of a gene expressed specifically in mesangial cells and elucidation of its function would be helpful in understanding the biological

25   characteristics of mesangial cells and the causes of diseases relating to mesangial cells, and in turn, treating or diagnosing diseases related to mesangial cells.

It has been reported that steroidal agents are effective for IgA nephropathy with an extreme proliferation of mesangial cells

30   (J.V.Donadio et al., J.Am.Soc.Nephrol. 8:1324-1332 (1997)). However, use of steroidal agents often causes serious side effects, and thus, aimless administration of steroidal agents to types of IgA nephropathy for which steroidal agents are ineffective, should be avoided. Therefore, a matching up with the severity of the tissue such as the

35   degree of proliferation of mesangial cells, and so on, is essential for steroid therapy. Accordingly, a renal function assay that can

simply and conveniently evaluate the degree of proliferation of mesangial cells was long desired.

The present inventors assumed that megsin protein gene expression increases in relation to the onset and progress of renal diseases, and in association thereto, megsin protein production increases and leads to a leakage of megsin protein into urine or blood, the amount of which may be related to the progression of disease state. To verify the mechanism, the inventors attempted to measure and compare the concentration or amount of megsin protein in various biological specimens, and thus, accomplished the present invention through the result that renal diseases related to megsin protein can be evaluated by measuring the concentration or amount of the megsin protein. Thus, the present invention relates to a method for evaluating renal functions, as well as to reagents or reagent kits for the method, which are as follows:

(1) a method for evaluating renal functions, which comprises measuring the megsin protein in a biological specimen;

(2) the method for evaluating renal functions of (1), which comprises measuring the megsin protein in a biological specimen, and comparing it with the megsin protein amount in a normal specimen;

(3) the method for evaluating renal functions of (1), wherein the biological specimen is urine;

(4) the method for evaluating renal functions of (1), wherein the megsin protein in the biological specimen is measured by an antigen-antibody reaction using an anti-megsin protein antibody;

(5) the method for evaluating renal functions of (4), wherein the anti-megsin protein antibody is a monoclonal antibody;

(6) a reagent for diagnosing renal functions, which comprises the anti-megsin protein antibody;

(7) the reagent for diagnosing renal functions of (6), wherein the anti-megsin protein antibody is a monoclonal antibody;

(8) a granule for detecting megsin protein in a biological specimen, wherein the granule comprises a solid granule to the surface of which an anti-megsin protein antibody is bound;

(9) the granule for detecting megsin protein of (8), wherein the solid granule is magnetic;

(10) the granule for detecting megsin protein of (8), wherein the relative density of the solid granule is not smaller than 1;

(11) the granule for detecting megsin protein of (8), wherein the anti-megsin protein antibody is a monoclonal antibody;

(12) a method for detecting megsin protein in a biological specimen, comprising the following steps of:

i) contacting the granule for detecting megsin protein with the biological specimen, said granule comprising a solid granule to the surface of which an anti-megsin protein antibody is bound;

ii) contacting the second anti-megsin protein antibody bound to a marker molecule with said granule for detecting megsin protein to which the biological specimen was contacted; and,

iii) detecting the marker molecule bound to the megsin protein through the second anti-megsin protein antibody;

(13) the method for detection of (12), wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are both monoclonal antibodies;

(14) the method for detection of (13), wherein the first anti-megsin protein antibody and the second anti-megsin protein antibody are antibodies having different recognition sites;

(15) the method for detection of (12), wherein the biological specimen is urine;

(16) the method for detection of (12), wherein the biological specimen is blood;

(17) a kit for detecting megsin proteins, which comprises the following elements:

a) magnetic solid granules to which anti-megsin protein antibodies can be bound,

b) anti-megsin protein antibodies, which are bound to said magnetic solid granules in advance, or can be bound to them indirectly, and

c) a magnet;

(18) the kit for detecting megsin proteins of (17), further comprising an anti-megsin protein antibody to which a marker molecule is bound.

[0010]

[Mode for Carrying Out the Invention]

As mentioned above, the megsin protein was isolated as a protein

encoded by a gene that is highly expressed in human kidney mesangial cells. The megsin protein of the present invention includes not only a protein having the amino acid sequence of SEQ ID NO: 2 (human megsin protein), but also proteins functionally equivalent thereto. For example, megsin protein homologues from species other than human can be mentioned as proteins that are functionally equivalent. Other examples are precursors and fragments of these homologues. Megsin protein-protease complexes can also be used as indicators of renal functions, as long as they can be detected within biological specimens. Megsin protein homologues of species other than humans can be used as indicators for the evaluation of renal functions in the respective species. Thus, they are useful to understand disease states in renal function disorder models.

[0011]

Megsin protein homologues from species other than human can be obtained, for example, as follows: First, the homologue cDNA is amplified by PCR utilizing a degenerative primer designed based on the nucleotide sequence of the cDNA encoding the human megsin protein of SEQ ID NO: 1 to correspond to the beginning and end of the ORF. In case where the amplified cDNA is not a full-length cDNA, its upstream nucleotide sequence can be determined by methods such as 5'RACE. For example, RT-PCR is conducted using the mRNA, which is extracted from cultured mesangial cells, as the template for rats. Degenerative primers used can be, for example, the oligonucleotides with the following nucleotide sequences. Part of the structure of the rat homologue megsin protein can be revealed by sequence determination of the clone obtained by such a PCR method.

Degenerative primer FY: GTGAATGCTGTGTACTTAAAGGCAANTGN/ SEQ ID NO: 3 (corresponding to 172VNAVYFKGK180)

Degenerative primer R21: AANAGRAANGGRTCNGC/ SEQ ID NO:4 (wherein R is A or G; corresponding to 357ADHPFLF363)

However, to obtain the 5'regions, it is necessary to conduct a degenerate PCR again by preparing gene specific primers from clone fragments of the rat megsin protein, since only a part of the cDNA can be amplified with these primer pairs. The nucleotide sequences of the primers used, which correspond to the N terminal amino acid

sequences of the megsin proteins are as follows:
Degenerative primer RM-CtermC1: ATGGCNTCNGCNGCNGCNGCNAAYGC/ SEQ ID NO:5 (wherein Y is T or C; corresponding to the sequence encoding the N-terminus of the megsin protein)

5   RM-MR-A2: CGACCTCCAGAGGCAATTCCAGAGAGATCAGCCCTGG/ SEQ ID NO: 6
RM-MR-A1: GTCTTCCAAGCCTACAGATTTCAAGTGGCTCCTC/ SEQ ID NO: 7
(Each of them is a rat megsin protein specific reverse primer)
    The 3' region of the cDNA encoding the rat homologue can be derived by inserting the obtained PCR product into the pGEM-T-easy

10 vector (Promega) to determine the nucleotide sequence. Finally, the nucleotide sequence of the full-length cDNA encoding the homologue can be determined by conducting 5'-RACE and 3'-RACE using a primer designed based on the nucleotide sequence obtained as above. Two gene specific   antisense   primers,   namely   RM-PRO1:

15 GCTCAGGGCAGTGAAGATGCTCAGGGAAGA/   SEQ   ID   NO:8   and   RM-PRO2:
CTGACGTGCACAGTCACCTCGAGCACC/ SEQ ID NO:9, can be used for the 5'-RACE for rats. On the other hand, gene specific sense primer RM-MR-S3: GAGGTCTCAGAAGAAGGCACTGAGGCAACTGCTGCC/ SEQ ID NO:10 can be used in the 3'-RACE. Mouse homologues can be isolated by the same method.

20 The sequence of rat and mouse megsin cDNA nucleotide sequence depicted in SEQ ID NO:15 (rat) and SEQ ID NO:17 (mouse) were determined according to the method above.
    [0012]
    The method used in the present invention to measure the megsin

25 protein is not restricted. For example, immunological assays, which make use of the immunological reaction between an antibody for the megsin protein and the megsin protein, are excellent in both specificity and sensibility. Furthermore, the megsin protein can be measured by the Western Blot method utilizing an antibody against

30 the megsin protein. Since the megsin protein is a protease inhibitor, it can be detected using its inhibition activity on protease as the indicator. Alternatively, the megsin protein can be assayed utilizing its affinity towards the protease.
    [0013]

35 The source or preparation method of the antibody, which is essential for immunological assays of the megsin protein, can be any

so long as the antibody can recognize the detection target, which is the megsin protein. Thus, antibodies to be used include polyclonal antibodies, monoclonal antibodies, mixtures thereof, etc. Furthermore, fragments containing the variable region of the antibody molecule are included. The antibody against megsin protein can be obtained, for example, as follows. For example, antibodies used in the present invention comprise those antibodies raised against the protein having the amino acid sequence of SEQ ID NO:2. The antibody (for example, polyclonal antibody or monoclonal antibody) or the antiserum against the megsin protein or partial amino acids sequence thereof can be prepared according to conventional methods for antibody or antiserum preparation using megsin proteins, oligopeptides comprising a partial amino acid sequence thereof, as well as fusion proteins, such as c-myc-(His)$_6$-Tag-megsin protein and MBP-megsin protein, as the antigens. For example, monoclonal antibodies can be prepared following the methods below. In cases where synthetic peptides with partial amino acid sequence are used as immunogens, it is generally convenient to use highly hydrophilic amino acid sequences that are specific to megsin protein. Such amino acid sequences include those shown in SEQ ID NO:11 to 14, which were used in the examples.

[0014]

The megsin protein of the present invention or a synthetic peptide having a partial amino acid sequence of the present megsin protein, is administered alone or together with a carrier or diluent to a warm-blooded animal at a site capable of producing antibodies. Synthetic peptides used as immunogens are in the form peptides bound to carrier proteins such as bovine thyroglobulin and keyhole limpet hemocyanin. To enhance the antibody productivity, the complete Freund's adjuvant or incomplete Freund's adjuvant can be administered together with the antigen. Immunization is performed once every 1 to 6 weeks, a total of about 2 to 10 times, in general. Warm-blooded animals to be used are, for example, monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, and domestic fowls, preferably mice or rats. Monoclonal antibody-producing cells can be prepared by selecting immunized warm-blooded animals, such as mice, in which an

antibody titer is detected, obtaining the spleen or lymph nodes from the animals 2 to 5 days after the final immunization, and fusing the antibody producing cells contained in these tissues with myeloma cells. Reacting the labeled megsin protein described below with the antiserum,

5   and measuring the activity of the label bound to the antibody enables measurement of the antibody titer in the antiserum.

[0015]

A monoclonal antibody of the present invention that does not crossreact with proteins other than megsin protein can be obtained

10   by selecting an antibody that recognizes epitopes specific to megsin protein. In general, an epitope specific to the protein comprises at least 7 or more continuous amino acid residues, preferably 10 to 20 amino acids in the amino acid sequence of the protein. Therefore, for example, a monoclonal antibody recognizing an

15   epitope comprising peptides having an amino acid sequence selected from the amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 16(rat), or SEQ ID NO: 18(mouse), and comprising at least 7 continuous amino acid residues can be a monoclonal antibody specific to the megsin protein. Conserved amino acid sequences among the amino acid

20   sequences of SEQ ID NO: 2, SEQ ID NO: 16(rat), and SEQ ID NO: 18(mouse) can be selected to choose epitopes common to the megsin protein family. If a region contains amino acid sequences specific to all sequences, a monoclonal antibody capable of recognizing different species can be selected.

25   An anti-MEGSIN protein monoclonal antibody can be separated and purified according to a separation and purification method for immunoglobulin, similar to the separation and purification of polyclonal antibodies. The known purification methods include, for example, salting out, alcohol precipitation, isoelectric point

30   precipitation, electrophoresis, adsorption and desorption method by ion exchangers (for example, DEAE), ultra centrifugation, gel filtration, or specific purification methods whereby an antibody is exclusively collected, for example, methods whereby an antigen is collected using an antigen binding solid phase or active adsorbent,

35   such as Protein A or Protein G, and thereafter the binding is dissociated to obtain the antibody.

[0016]

Monoclonal antibodies and polyclonal antibodies obtained in such a manner that recognize the megsin protein of the present invention, can be used for the diagnosis and treatment of diseases relating to mesangial cells. Examples of methods that use these antibodies to measure the megsin protein include a sandwich assay, which comprises reacting the megsin protein with an antibody binding to an insoluble carrier and a labeled antibody and detecting MEGSIN protein in the sandwiched complex produced by the reaction, or a competitive method comprising competitively reacting labeled human urine-derived megsin protein and human urine-derived megsin protein in a sample with an antibody to measure human urine-derived megsin protein in the sample based on the labeled antigen amount that reacted with the antibody.

The assaying of human urine-derived megsin protein by the sandwich method is conducted by a 2 step method in which an immobilized antibody is reacted with human urine-derived megsin protein, unreacted materials are completely removed by washing, and a labeled antibody is added to form the immobilized antibody-labeled human urine-derived MEGSIN protein antibody complex, or one step method in which the immobilized antibody, the labeled antibody, and human urine-derived MEGSIN are mixed at the same time.

[0017]

Examples of insoluble carriers used for the assay include, for example, polystyrene, polyethylene, polypropylene, polyvinyl chloride, polyester, polyacrylate, nylon, polyacetal, synthetic resin such as fluoride resin, etc., polysaccharides such as cellulose, agarose, etc., glass, metals, etc. The form of the insoluble carrier can be varied and includes a shape like a particle, tray, sphere, fiber, stick, board, container, cell, test tube, etc. The antibody-adsorbed carrier should be stored in a cool place in the presence of appropriate preservatives, such as sodium azide.

On the other hand, solid granules are known as reagents used to evaluate the fertility of human sperm cells. These granules comprise on their surfaces monoclonal antibodies that specifically react with human sperm cells having undergone the acrosome reaction (Japanese Patent No.2651249). This granule used for sperm fertility detection is a solid granule to which a monoclonal antibody that

specifically reacts with human sperm cells having undergone the acrosome reaction is bound. The fertility of human sperm cells can be evaluated by reacting sperm cells with the granules and counting sperm cells bound to the granules. The fertility can be tested readily

5   and safely without the use of complicated techniques like radioactivity, fluorescence, and so on. Samples of small quantities can be assayed by using magnetic solid granules since the granules can be aggregated easily by using a magnet. The present inventors found out that it is possible to detect megsin protein more easily

10 and accurately by applying this technique.
    [0018]

    Antibodies can be immobilized by known chemical binding or physical adsorption methods. Chemical binding methods include, for example, a method using glutaraldehyde, the maleimide method using

15 N-succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate, N-succinimidyl-2-maleimidoacetate, etc., and the carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, etc. Further examples are, the maleimidobenzoyl-N-hydroxysuccinimidoester method, the

20 N-succimidyl-3-(2-pyridyldithio)propionate method, the bisdiazolated benzidine method, and dipalmityllysine method. Alternatively, a complex, which is prepared by reacting two different antibodies having different epitopes with the substance to be detected, can be captured with a third antibody immobilized

25 by the above method.
    [0019]

    The label is not limited, so long as it is useful in immunoassays. Specifically, enzymes, fluorescent substances, luminescent substances, radioactive substances, metal chelates,

30 etc. can be used. Preferable labeling enzymes are, for example, peroxidase, alkaline phosphatase, β-D-galactosidase, malate dehydrogenase, *Staphylococcus* nuclease, delta-5-steroid isomerase, α-glycerol phosphate dehydrogenase, triosephosphate isomerase, horseradish peroxidase, asparaginase, glucose oxidase,

35 ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase, etc. Preferable

fluorescent substances include, for example, fluorescein isothiocyanate, phycobiliprotein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, and orthophthalaldehyde. Preferable luminescent substances include, for example, isoluminol, lucigenin, luminol, aromatic acridiniumester, imidazole, acridinium salt and its modified ester, luciferin, luciferase, and aequorine. Preferable radioactive substances include, for example, ^{125}I, ^{127}I, ^{131}I, ^{14}C, ^{3}H, ^{32}P, ^{35}S, etc.

[0020]

Methods for binding the above labels are known. Specifically, direct and indirect labeling can be used. The commonly used direct labeling method is the method in which an antibody or an antibody fragment is chemically covalent-bound to a label using a cross-linking agent. Cross-linking agents include N,N′-orthophenylenedimaleimide, 4-(N-maleimidomethyl) cyclohexanoate N-succinimide ester, 6-maleimidohexanoate N-succinimide ester, 4,4′-dithiopyridine, and other known crosslinking agents. The crosslinking agent can be reacted with enzymes and antibodies by known methods depending on the characteristics of the cross-linking agent. An example of the indirect labeling method comprises binding an antibody to a low molecular weight hapten such as biotin, dinitrophenyl, pyridoxal, or fluorescamine, and indirectly labeling the antibody with the binding partner to the hapten. Avidin and streptavidin can be used as a recognition ligand for biotin, whereas dinitrophenyl, pyridoxal, or fluorescamine are labeled with antibodies recognizing these haptens.

Horseradish peroxidase can be used as an enzyme for labeling antibodies. This enzyme is useful because it can react with many substrates and be easily bound to antibodies by the periodate method. Occasionally, antibody fragments, for example, Fab′, Fab, $F(ab')_2$ are used as antibodies. Both polyclonal and monoclonal antibodies can be labeled with an enzyme by the same method. Enzyme-labeled antibodies obtained using the above crosslinking agent can be purified by known methods such as affinity chromatography, etc. to serve as more sensitive immunoassay systems. Purified enzyme-labeled antibodies are stored with a preservative such as

thimerosal and a stabilizer such as glycerol. Labeled antibodies can be lyophilized and stored in the cool and dark place for a long time.

[0021]

When the label is an enzyme, its substrate and, if necessary, a coloring agent is used for measuring its activity. When peroxidase is used as an enzyme, H_2O_2 is used as the substrate solution, and 2,2'-azino-di-[3-ethylbenzothiazolinesulfonic acid] ammonium salt (ABTS), 5-aminosalicylic acid, orthtophenylenediamine, 4-aminoantipyrine, or 3,3', 5,5'-tetramethylbenzidine, etc. are used as coloring agents. When alkaline phosphatase is used as the enzyme, orthonitrophenylphosphate, paranitrophenylphosphate, etc. can be used as substrates. When β-D-galactosidase is used as the enzyme, fluorescein-di-(β-D-galactopyranoside), 4-methylumbelliferyl-β-D- galactopyranoside, etc. can be used as substrates. The present invention also includes an immunoassay reagent for megsin comprising labeled or immobilized monoclonal or polyclonal antibodies. The present invention further includes a kit comprising this reagent and an indicator for detection labels and a control sample, etc.

Any biological sample such as body fluid including blood plasma, serum, blood, urine, tissue fluid, or cerebrospinal fluid, etc. can be used as samples for measuring the megsin protein of the present invention, as long as they contain megsin protein or its precursor or a fragment. Among these biological samples, megsin protein can be detected with high frequency especially in urine, accompanied by a proliferation and activation of mesangial cells. Therefore, measurement of megsin in urine is useful as a marker for mesangial proliferative nephropathy, such as IgA nephropathy.

[0022]

Evaluation of renal functions as used herein means to comprehend the condition of mesangial cells, which is an important cell constructing kidney tissue, and to determine the presence or severity of a renal disease causing abnormalities in mesangial cells. Renal disease as used herein refers to any disease causing an abnormality

in mesangial cells. More specifically, IgA nephropathy, acute glomerulonephritis, focal sclerosing glomerulopathy, membranoproliferative glomerulonephritis, diabetic nephritis, lupus nephritis, and so on, can be mentioned as such diseases. The condition of renal functions affected by these renal diseases can be evaluated by the method of the present invention. Among the above diseases, the present method for evaluating renal function is especially useful as a marker for mesangial cell proliferative nephropathy, such as the IgA nephropathy. Moreover, the method of the present invention for evaluating renal function may be applied not only to determine the existence or severity of a renal disease, but also to evaluate a therapeutic effect or make a prognosis. In addition, the method of the present invention for evaluating renal function enables the exclusion of diseases not ascribable to the proliferation of mesangial cells like acute pyelonephritis, chronic pyelonephritis, minimal-change nephritic syndrome, chronic glomerulonephritis, renal amyloidosis, etc.

[0023]

To evaluate renal functions according to the present invention, a biological specimen from an individual whose renal function should be tested is obtained and the concentration of megsin protein therein is measured following the above-mentioned methods. Preferably, the amount of megsin protein is determined from the measured concentration and the volume, and compared to that of a normal healthy person. To calculate the amount of megsin protein using urine as the sample, all urine of a day is pooled and the amount of urine is measured. Thereby, it is possible to determine the amount of megsin protein in a day's urine. Alternatively, it is possible to presume the amount by creatinine correction, even when the urine is sampled occasionally. The term "creatinine correction" refers to a method to correct the influence of dilution (or concentration) of the object component due to the changes in urine amount based on the creatinine concentration. The creatinine amount excreted daily into the urine is constant. Based on this fact, the percentage occasionally collected urine occupies in the daily total excretion is calculated from the creatinine concentration, and the concentration of the object

component in the same urine is converted into the total excretion of a day. Alternatively, correction methods generally used in the diagnosis of renal functions such as weight correction is applied to presume the amount in blood. Weight correction is a method, wherein

5　the amount of blood component is calculated based on the volume presumed from the weight of the individual from whom the blood was taken.

Alternatively, changes in renal function can be also followed in a time course without having to convert into amounts by observing

10　the changes of megsin protein concentration in a biological specimen derived from a certain individual. Alternatively, an abnormality in renal function can be determined by configuring the normal values for megsin protein concentration in body fluids of specific species or groups like the race, and then, comparing the megsin protein

15　concentration (or amount) of an individual with the normal value.

The biological specimen used in the present invention can be urine or blood. Urine is more preferable since it can be collected in a noninvasive manner and is at the same time a sample that reflects the condition of renal functions directly. Although blood must be

20　collected with some invasiveness, since megsin protein is a protein specific to the kidney, an abnormality of the measured value closely relates to an abnormality of renal function. Thus, a high specificity can be expected if the amount of megsin protein in blood and urine is used as indicators of renal function. Compared to the currently

25　widely used method of screening for renal function disorder using urinary proteins as indicators, a more specific screening method with a superior sensibility can be expected by utilizing the megsin protein as the indicator. A method for evaluating renal functions by measuring the megsin protein in urine based on the immunological

30　method for measuring the megsin protein using a monoclonal antibody will be explained in detail below.
[0024]
(A) Preparation of the antibody
(1) Immunization of the animal and preparation of antibody-producing

35　cells

The immunization of the animal is conducted, for example, as

follows. A mammal, such as a rat, mouse, and so on, is immunized with the human megsin protein purified according to conventional methods (for example, T.Miyata et al., J.Clin.Invest., 120:828-836 (1998)). It is preferable to immunize animals of the same strain as the animal

5   strain from which the permanently proliferating cell used during cell fusion is obtained. For example, mice at the age of 8 to 10 weeks are preferable. Mice of both genders will do. The immunization is conducted as follows: (1) the purified human megsin protein is mixed to an emulsion with an appropriate adjuvant (for example, Freund's

10 complete adjuvant, aluminum hydroxide gel-pertussis vaccine, etc.), and (2) administered to the animal subcutaneously, intraperitoneally, intravenously, etc. Repeating this immunization process 2 to 5 times, with an interval of 1 to 2 weeks. The final immunization is accomplished by an intraperitoneal administration of 0.5 to 2 µg human

15 megsin protein to the animal. Polyclonal antibodies are obtained from the body fluid of the thus immunized animal. 3 to 7 days after each immunization process, blood is collected from venus plexus of the eyeground, and the antibody titer of the serum is measured by the protein A rosette assay (Eur.J.Immunol., 4:500-507 (1974))

20 described below. The antibody or the antibody-producing cell is harvested as the antibody titer had elevated enough.

[0025]

The protein A rosette assay is, for example, performed as follows: (1) 72 well Terasaki Plate (Falcon) is coated with the human

25 erythroblast cell line K562 (Japanese Cancer Research Resources Bank (JCRS)); (2) samples diluted with PBS (sodium dihydrogen phosphate 2.90 g, potassium monohydrogen phosphate 0.20 g, sodium chloride 8 g, potassium chloride 0.2 g, distilled water 1 L)are added; (3) the plate is left standing for 30 minutes at 37°C in a CO_2 incubator; (4)

30 the wells are washed with PBS; (5) sheep erythrocytes coated with protein A (Amersham Pharmacia Biotech)are added; and, (6) the formed rosettes are observed with a microscope.

Antibody-producing cells are harvested from the above human megsin protein immunized animal. Antibody-producing cells can be

35 obtained from the spleen, lymph nodes, peripheral blood, and so on. However, the spleen is especially preferred. For example, 3 to 4 days

after the final immunization, the spleen is excised aseptically, cut in a Minimal Essential Medium (MEM) (Nissui Pharmaceutical), detach tissues and cells with a tweezer, and after centrifugation at 1,200 rpm for 5 minutes the supernatant is discarded. Then the pellet is treated with Tris-HCl buffer (pH 7.65) for 1 to 2 minutes, and erythrocytes are eliminated. After washing 3 times with MEM, spleen cells used for cell fusion are obtained.

[0026]

(2) Preparation of permanently proliferating cells

Any cell with permanent proliferation ability can be used as the permanently proliferating cell for the fusion. However, myeloma cells are generally used. It is preferable to use cells derived from the same specie as that from which the antibody-producing cell was obtained. For example, in the case of mouse, the following cell lines are known as 8-azaguanine resistant mouse (BALB/c)-derived myeloma cell lines.

P3-X63Ag8-U1 (P3-U1) (Current.Topics in Microbiol.Immunol., 81:1-7, (1978)),

P3/NS1/1-Ag4-1 (NS-1) (Eur.J.Immunol., 6:511-519, (1976)),

SP2/0-Ag14 (SP-2) (Nature, 276:269-270, (1978)),

P3-X63-Ag8653 (653) (J.Immunol., 123:1548-1550, (1979), and

P3-X63-Ag8 (X63) (Nature, 256:495-497, (1975))

These permanently proliferating cell lines are subcultured in 8-azaguanine media (normal media, RPMI-1640 medium supplemented with glutamine (1.5 mM), 2-mercaptoethanol (5×10^{-5} M), gentamicin (10 µg/mL) and fetal calf serum (FCS, CLS) (10%), supplemented with 8-azaguanine (15 µg/mL)), and passaged to normal media 3 to 4 days before cell fusion to ensure a cell count of 2×10^7 cells at the day of cell fusion.

[0027]

(3) Cell fusion

Fusion of cells is, for example, performed as follows. The antibody-producing cell obtained in (1), and the permanently proliferating cell prepared in (2) are mixed at a cell rate of 5 to 10:1 after washing well with MEM or PBS. After centrifugation at 1,200 rpm for 5 minutes, the supernatant is removed and the precipitated

cells are detached well.   0.1 to 1.0 mL/10^8 cells are added to a mixture of 1 to 4 g polyethylene glycol-1000 (PEG-1000), 1 to 4 mL MEM, and 0.5 to 1.0 mL dimethyl sulfoxide, and the mixture is kept at 37°C while stirring to fuse the cells.   Then, the mixture is diluted by adding 3 mL MEM few times every 10 minutes to a total volume of 50 mL MEM to quench the cell fusion reaction.   Next, the supernatant is discarded after centrifugation (1,500 X 5 min), cells are gently tapped, 100 mL normal medium (RPMI-1640 media, 10% FCS) is added, and the cells are suspended by gentle pipetting with a measuring pipette.   100 μL suspension is dispensed to each well of a 96 well culture plate, and incubated at 37°C for 3 to 5 days in an 5% CO_2 incubator.   100 μL of HAT medium (normal medium supplemented with hypoxanthine (10^{-4} M), thymidine (1.5 X 10^{-5} M), and aminopterin (4 X 10^{-7} M)) is added to each well of the culture plate, and incubated further for 3 days.   Thereafter, half of the culture supernatant is discarded and the same amount of HAT medium is added thereto every three days.   The incubation is continued for about 2 weeks at 37°C in a 5% CO_2 incubator.

[0028]

(4) Screening and cloning of the hybridoma

Supernatants are removed from wells in which fusion cells are observed to grow in colonies, same amount of HT medium (HAT medium without aminopterin) is added, and the cells are incubated for 4 days. Part of the culture supernatant is harvested to measure the antibody titer against megsin protein by the aforementioned protein A rosette assay.   The antibody titer can be also assayed by other methods like the following.   For example, 1) by adding the hybridoma culture supernatant to a solid phase (e.g. microplate), where the megsin protein antigen is attached directly or together with a carrier; 2) adding anti-immunoglobulin antibody (if the cell for cell fusion is derived from mouse, anti-mouse immunoglobulin antibody is used) labeled with radioactive substances or enzymes, or protein A; and 3) detecting the anti-megsin protein monoclonal antibody bound to the solid phase.   The antibody titer can be also assayed by: 1) adding the hybridoma culture supernatant to a solid phase, where an anti-immunoglobulin antibody or protein A is attached; 2) adding

megsin protein labeled with radioactive substances or enzymes, and 3) detecting the anti-megsin protein monoclonal antibody bound to the solid phase.

 Cloning is repeated 4 times by limiting dilution method for
5 wells in which the production of antibodies that react to the megsin protein was observed, and cell lines, which show stable megsin protein antibody titers are selected as anti-megsin protein monoclonal antibody producing hybridoma cell lines.

 [0029]
10 (5) Preparation of the monoclonal antibody

 The monoclonal antibody is produced by incubating the thus obtained hybridomas *in vitro* or *in vivo*. In case where the incubation is conducted *in vivo*, the hybridoma is transplanted to appropriate animals. It is preferable to use the same kind of animal as that from
15 which the spleen cells for cell fusion were taken. For example, 2 to 4 X 10^6 cells of anti-megsin protein monoclonal antibody producing hybridoma cells obtained in (4) is administered intraperitoneally to each 8 to 10 week old BALB/c female mouse treated with pristane (reared for 2 weeks after intraperitoneal administration of 0.5 mL
20 2,6,10,14-tetramethylpentadecane-pristane). Subsequently, after 2 to 3 weeks, the abdomen of the mouse enlarges due to the accumulation of ascites, which contain the monoclonal antibody at a high concentration, in the visceral cavity of the mouse. The ascites are then collected from the mouse, centrifuged (3,000 rpm X 5 min) to
25 remove solid matters, and the IgGs are purified. Alternatively, the hybridoma is cultured *in vitro* in a serum-free and an appropriate amount of antibody secretes to the supernatant. The ascites or the culture supernatant is salted out with 50% ammonium sulfate, and then dialyzed for 1 to 2 weeks with PBS. The dialyzed fraction is passed
30 through a protein A Sepharose column, and the IgG fraction is collected to obtain a purified monoclonal antibody.

 The isotype of the antibody is determined according to the Ouchterlony method (double immunodiffusion) ("Menekigaku jikken nyumon, Seibutsugaku jikkenhou 15" (A laboratory manual for immunology,
35 Biochemical experimentation 15), Gakkai Shuppan Center, p74 (1981)). The quantity of the protein is calculated by the Folin method and

by the absorbance at 280 nm (1.4 (OD_{280}) ≒ immunoglobulin 1 mg/mL).

[0030]

(6) Characterization of the monoclonal antibody

The characterization of the monoclonal antibody obtained above can be performed by, for example, (1) immunoprecipitation method utilizing human lymphocyte derived cell lines, such as HSB-2, K562, and so on, the cell surface of which is labeled with iodine (J.Immunol., 138: 2850-3855 (1987)); (2) enzyme immunoassay (ELIZA) (J.Immunol., 142: 2743-2750 (1989)); and such.

[0031]

(7) Preparation of labeled monoclonal antibody

The obtained purified monoclonal antibody may be enzyme labeled by methods like glutaraldehyde method (Immunochem., 6: 43 (1969)), periodic acid method (J.Histochem.Cytochem., 22: 1084 (1974)), maleimide method (J.Biochem., 79: 233 (1976)), pyridil disulfide method (Biochem.J., 173: 723 (1978)), etc.

For example, the periodic acid method is performed as follows: (1) periodic acid (38.5 mg/mL) is added to the peroxidase solution (4 mg/mL) while stirring; (2) reacted at room temperature for 20 minutes; (3) the buffer is exchanged using PD-10 (Amersham Pharmacia Biotech) substituted with 1 mM acetate buffer (pH 4.5); (4) 40 μL of 0.2 M sodium hydroxide is added; (5) 10 mg of monoclonal antibody dialyzed with 10 mM carbonate buffer is added(pH 9.5); (6) react at room temperature for 2 hours; (7) after the reaction is completed, the mixture is cooled on ice; (8) 100 μL solution of sodium boron hydride (4 mg/mL) is added and reacted for 2 hours; (9) the reaction solution is changed to PBS using PD-10; (10) centrifuged at 3,000 rpm for 30 minutes; (11) the supernatant is gel filtrated using Sephacryl S200HR26 X 30 (Amersham Pharmacia Biotech); (12) the labeled monoclonal antibody fraction is obtained by detecting the absorbance at 403 and 280 nm; (13) bovine serum albumin (10 mg/mL) is added to the obtained fraction; (14) stored at -20°C, and diluted with PBS-Tween (Trademark) 20 before usage.

[0032]

(B) Preparation of granules for detection

The granules for detection used herein may be prepared by

physically or chemically binding anti-megsin protein monoclonal antibody to appropriate granules, for example, gel for chromatography. The method used in the present invention for binding the antibody to chemically activated granules is a preferable method due to the expected binding stability. More specifically, the method for binding the antibody used in the present invention to granules that are actively tosylated by p-toluenesulfonyl chloride can be given.

Granules to be used are made of glass, agarose, Sepharose, agarose filled porous diatomite, hydrophilic copolymerized acrylic gel, polystyrene, and so on. Use of magnetic granules enables collection of the granules with the use of magnet, and so on, thereby allowing detection of small quantity samples. For example, by placing substances capable of being magnetic (e.g. Fe_2O_3) in the core of the granules, it is possible to make the granules superparamagnetic. Such granules are commercially available as solid phases for immunological analyses. The granule may take any appropriate form such as a sphere, amorphous fracture, etc., however, sphere shaped granules are preferred. There is no limitation on the particle size of the granule, and an average particle size, for example, of 5 to 1000 μm can be given. Furthermore, the use of granules with larger specific gravity than that (about 1) of the reaction solution facilitates collection of the granules, giving an effect similar to that when magnetic granules are used. Furthermore, the use of granules with larger specific gravity is more advantageous for antibodies whose binding is easily disassociated, because gentle centrifugation conditions are possible when collecting the granules.

Anti-megsin protein antibodies can be bound to the granules not only by direct binding but also by indirect binding. For example, if a mouse monoclonal antibody is used as the antibody, an antibody that recognizes mouse IgG is attached to the granule and the mouse antibody can be bound to the granule indirectly. Such antibodies are called secondary antibodies. Indirect binding can be also accomplished by utilizing protein A or protein G, which bind to the constant region of immunoglobulin, or by capturing avidin-attached granules with biotinylated antibodies, and so on. To chemically bind the secondary antibody, protein A, or protein G to the granule, it

is preferable to activate the granules before binding. As an activation method of the granule, any appropriate activation method used to conjugate proteins to the granule can be selected. Such activation methods include, the tosyl chloride method, bromcyane

5 method, bromacetyl method, glutaraldehyde method, etc. There are also commercially available activated granules. Activation and binding of these activated granules to proteins, such as secondary antibodies, protein A, protein G, and such, can be performed according to conventional methods.

10   [0033]

In addition, granules conjugated to secondary antibodies, protein A, protein G, and so on are also commercially available. Granules mentioned below are known as commercially available granules.

15   Dynabeads™ M-450, M-280 provided by Veritas, and imported by Dynal Japan

Sheep anti-mouse IgG coated type

Goat anti-mouse IgG coated type

Sheep anti-rat IgG coated type

20   Sheep anti-rabbit IgG coated type

Polyscience Inc.

Goat anti-mouse IgG (H&L) carboxylate beads

Goat anti-rabbit IgG (H&L) carboxylate beads

Protein A carboxylate beads

25   Goat anti-rabbit IgG (H&L) micromagnet particle

Goat anti-rabbit IgG (H&L) micromagnet particle

Protein A micromagnet particle

Sheep anti-mouse IgG (H&L) micromagnet particle

The binding of the present antibody to the granules above is

30 conducted as follows: (1) unspecific binding is prevented by treating the granules suspended in appropriate media with a protein solution, and (2) mixing the ascites containing the antibody or the purified antibody solution.

[0034]

35 (C) Detection method

The detection method of the present invention is accomplished

by collecting blood or urine from the subject, and using the supernatant after centrifugation as the specimen. The centrifugation of urine is done to separate the precipitate, and the supernatant of urine left standing still, or urine from which the precipitate is removed by filtration can be also used. Specimen diluted with the detection granule prepared above and the labeled antibody obtained in (7) are mixed, and then, incubated at room temperature for 2 hours. After the reaction has come to an end, the mixture is washed, and the substrate solution is added for chromogenic reaction. Then, the mixture is centrifuged to remove the granules, the supernatant is transferred onto a microplate, and the absorbance is measured. A specimen from a normal healthy person is also assayed according to the same method, and the absorbance is compared. Mere comparison of the concentration of the megsin protein, as well as a comparison of the absolute quantity of megsin protein in the body fluid, which is obtained by multiplying with the volume of body fluid of the individual, or comparisons based on of other similar corrected values might be performed.

[0035]

(D) Kit

The materials used to perform the experiment above can be supplied as a kit. The kit may comprise a detection granule, to which the antibody is conjugated as described above, and a magnet. It may also include an antibody labeled with a marker molecule. Further, the kit of the present invention may include test tubes, centrifugation tubes, other similar containers, pipettes or other suction instruments, and microscopes. Furthermore, enzyme substrates necessary for detecting the label, and positive or negative standard samples may be combined. Solid granules and antibodies, which are the raw material for the detection granules mentioned above, may be included instead of the detection granules. The invention is described in detail with reference to the examples below, but it is not to be construed as being limited thereto.

[0036]

[Examples]

[Example 1] Production of polyclonal antibodies against synthetic

peptides of the megsin protein

Polyclonal antibodies against megsin protein were produced by using regions having low identity with other members of the serpin family and which are hydrophilic as the immunogens. Peptides having the amino acid sequences shown below with a cystein attached to the N-terminus or the C-terminus were synthesized by solid phase methods.

Position from N-terminus	Amino acid sequence	SEQ ID NO/No.
16- 30	CFREMDDNQGNGNVFF	11/1
72- 86	CSQSGLQSQLKRVFSD	12/2
339-354	ATGSNIVEKQLPQSTLC	13/3
342-356	CSNIVEKQLPQSTLFR	14/342

Each of these synthetic peptides were bound to bovine thyroglobulin (Sigma) using N-(6-maleimidocaproyloxy)succinimide (Dojindo Molecular Technologies Inc.), were dialyzed against 0.85% NaCl solution, and were mixed well with the adjuvant (Difco) to emulsion, and were administered subcutaneously to a rabbit. 3 weeks after priming (20 μg/rabbit), second immunization (50 μg/rabbit) was carried out, and thereafter, four immunizations (50, 100, 200 μg/rabbit) were performed every two weeks. Freund's complete adjuvant was used for the priming, and thereafter, incomplete Freund's adjuvant was used. After 41 days and 55 days, the antibody titer of the serum was evaluated by enzyme-linked immunosorbent assay (ELISA) to confirm that the serum obtained by blood sampling reacts with synthetic peptide. To conduct primary reaction, 100 μL of continuously diluted antiserum was added to each well of the 96 well plate, in which 50 ng/well antigen had been immobilized. After washing, the HRP conjugated goat anti-rabbit IgG (Cappel) was reacted against it in the second reaction. After washing, the coloring reaction was done using orthophenylenediamine (Wako Pure Chemical Industries, Ltd.) as the substrate and absorbance was measured at 492 nm. An increase in antibody titer was confirmed.

[0037]

[Example 2] Purification of polyclonal antibodies against synthetic

peptides of megsin protein

The polyclonal antibodies against each synthetic peptide of megsin protein were purified by immunoaffinity chromatography according to conventional methods (Cell Engineering supplement

5   "Jikken Protocol Series Kou-peptide Jikken protocol (Experimental protocol series: Anti-peptide experimental protocol)", Shujun-sha, Japan). More specifically, the method was carried out as follows: 1) an affinity column was prepared by immobilizing each synthetic peptide to FMP (2-fluoro-1-methylpyridinium toluene-4-sulfonate)

10   activated Cellulofine (Seikagaku Corp.); 2) after the rabbit serum showing increased antibody titer was diluted with PBS (-), the antibody was purified by affinity using the peptide column. It was demonstrated that the obtained purified antibody is specific to the megsin protein, by confirming its reaction against megsin protein

15   fusion protein by Western blotting (Fig. 1 to 3).
     [0038]

     [Example 3] Studies on reactivity of the rabbit polyclonal anti-megsin peptide IgG

     The reactivity of the rabbit IgG, which was produced using

20   megsin protein as the immunogen, was studied using the following various proteins as antigens. MBP-megsin protein and His-megsin protein were prepared as follows: 1) the coding region was amplified by PCR based on the nucleotide sequence of megsin gene described in SEQ ID NO: 1; 2) the amplified products were inserted into maltose

25   binding protein fusion protein expression vector pMAL-c (New England Biolab) to obtain vectors that express the fusion protein of MBP and megsin protein; 3) E.coli were transformed with this vector; and then, 4) the MBP-megsin protein was obtained by purifying the expression product from the disrupted cell solution by affinity chromatography

30   utilizing amylose, and such. To obtain His-megsin protein, a fusion protein expression vector containing aforementioned megsin gene in the vector ptrcHisA (Invitrogen), which expresses the inserted gene as a fusion protein with a histidine tag, was constructed. This vector was transformed into E.coli JM109, and following the induction of

35   expression by IPTG, the His-megsin protein was obtained as a fusion protein purified by nickel column and such from the disrupted cell

solution.

The megsin protein(+)virus-infected silkworm body fluids refers to body fluids of a silkworm, which is infected with megsin gene introduced virus according to known methods (J.General Virology 78: 3073-3080 (1979)). On the other hand, megsin protein(-)virus-infected silkworm body fluids refers to body fluids of a silkworm, which is infected with a virus without the megsin gene. The gene encoding megsin necessary for designing these megsin recombinants can be synthesized by PCR using mRNA of the mesangial cell as the template, or obtained from the megsin gene cloning vector (Accession No. FERM BP-6518) constructed by the inventors.

Reactivity against plasminogen activator inhibitor-1 (PAI I), MBP-plasminogen activator inhibitor-2 fusion protein (MBP-PAI II), and so on was also observed. These proteins were selected to confirm the cross-reactivity of the antibody, and share some extent of homology with the megsin protein.

MBP-megsin protein,

His-megsin protein,

MBP-PAI II,

PAI I,

megsin protein expressed in CHO cells,

megsin protein (+) virus-infected silkworm body fluid,

megsin protein (-)virus-infected silkworm body fluid,

non-virus-infected silkworm body fluid, and

human serum albumin.

[0039]

Each protein solution was treated with same amount of sample buffer (0.25% Tris-HCl, 2% SDS, 30% glycerin, 10% β-mercaptoethanol, 0.025% Bromophenol Blue) (Daiichi Pure Chemicals), heated at 100°C for 5 minutes, and the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli,U.K., Nature 227: 680-685 (1970)). The proteins separated by SDS-PAGE was blotted on poly vinylidene difluoride (PVDF) membrane (BioRad) with a constant voltage of 100 V for 1 hour, using the blotting solution (25mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). Following the washing of the blotted PVDF membrane with distilled water, the membrane was blocked

for 3 hours in TTBS solution containing 5% Block Ace. After washing
the PVDF membrane with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween20,
pH 7.5), it was reacted at 4°C, overnight with the primary antibody,
rabbit polyclonal anti-megsin peptide IgG, diluted in TTBS. Then it
5   was detected with Amplified Alkaline Phosphatase Immuneblot Kit
(BioRad). That is, it was first incubated for 1 hour with TTBS diluted
biotinylated goat anti-rabbit IgG at room temperature, then reacted
with streptavidin - biotinylated alkaline phosphatase complex, which
was prepared in advance by incubating streptavidin and biotinylated
10   alkaline phosphatase for 1 hour at room temperature. The PVDF
membrane was washed in TTBS, and then incubated with the substrate
(nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate,
p-toluidine salt solution) for about 30 minutes at room temperature
to visualize the antibody bound to the primary antibody. The reaction
15   was quenched by reacting with a sufficient amount of distilled water.
[0040]
[Example 4] Enzyme labeling of affinity purified anti-megsin peptide
IgG
The affinity purified anti-megsin peptide IgG obtained in
20   Example 2 was labeled with alkaline phosphatase using a commercial
labeling kit (AP labeling kit: Boehringer-Mannheim) following the
instructions in the kit. Biotinylation was performed with another
labeling kit (ECL protein biotinylation module: Amersham Pharmacia
Biotech) following the instructions.
25   [0041]
[Example 5] Measurement of urinary megsin protein by ELISA
Urine from 16 normal healthy persons, 24 IgA nephropathy
patients, and 24 minimal-change nephritic syndrome patients were
centrifuged to exclude the insoluble material. This was then
30   concentrated using an ultrafilter, with a molecular weight cut-off
of 5,000 (Ultarafree, Millipore). Each well of the 96 well ELISA plate
was coated with rabbit polyclonal anti-megsin peptide-2 IgG. 120 µL
PBS (-) was poured into each well, then 120 µL concentrated urine
was added thereto and was left standing overnight at 4°C. The wells
35   were washed with PBS (-), and Block Ace (Dainippon Pharmaceutical) was
added to conduct blocking for 2 hours at room temperature. The wells

were then washed with PBS (-) containing 0.05%(w/v) Tween20 (Wako Pure Chemical), alkaline phosphatase labeled rabbit polyclonal anti-megsin peptide-1 antibodies were added, and left standing for an hour at room temperature. After washing with Tween-PBS, 100 μL

5 of coloring substrate solution (1 mg/mL orthophenylenediamine (Wako Pure Chemical) - 1 M diethanolamine (Wako Pure Chemical) buffer (pH 9.8)) was added to each well. After reacting at room temperature, the reaction was quenched with 50 μL/well 1 N sodium hydroxide, and the absorbance at 405nm was measured with a microplate reader (Spectra

10 MAX 250, Molecular Devices). The megsin protein concentration in the urine was determined from the calibration curve. The creatinine concentration in the same urine specimen was determined with the commercially available creatinine-measuring reagent (measuring reagent for automated analyzer "Dia"-Crea, Dia Pharmaceutical), and

15 the amount of megsin protein was corrected as creatinine ratio. The result is shown in Fig. 4. A significant difference in megsin concentration was observed between that of the normal healthy persons and patients of IgA nephropathy (p<0.001), or between that of IgA nephropathy patients and minimal-change nephritic syndrome

20 patients(p<0.001).

Since a significant difference between normal healthy persons and IgA nephropathy patients was observed, onset of renal disorders could be confirmed by measuring urinal megsin protein. Furthermore, due to the significant difference observed between patients of IgA

25 nephropathy and minimal-change nephritic syndrome, it was revealed that these diseases could be distinguished by measuring urinal megsin protein according to the present invention. These diseases cannot be distinguished by conventional screening methods using urinary protein as indicators, because positive results would be shown for

30 both diseases.

Furthermore, in comparisons of urinary megsin protein concentration without creatinine correction, although megsin protein concentration tends to be higher for patients with renal disorders, it seemed difficult to prove a statistically significant difference

35 with a small number of examples. Thus, it is preferable to compare the amounts by methods like creatinine correction and such, when

implementing the measurement of megsin protein in urine according to the present invention.

[0042]

[Example 6] Detection and assay of urinary megsin protein by Western blotting

Urine from 8 normal healthy persons and 9 IgA nephropathy patients were centrifuged, after discarding insoluble material, the solution was concentrated with an ultrafilter with a molecular weight cut-off of 5,000 (Ultrafree, Millipore). The concentrated urine was treated with same amount of sample buffer (0.25% Tris-HCl, 2% SDS, 30% glycerin, 10% β-mercaptoethanol, 0.025% Bromophenol Blue) (Daiichi Pure Chemicals), heated at 100°C for 5 minutes, and separated in a gradient gel with a gel gradient of 4 to 20% (Daiichi Pure Chemicals) by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, U.K. (1970) Nature 227, 680-685). The protein separated by SDS-PAGE was blotted on poly vinylidene difluoride (PVDF) membrane (BioRad) with constant voltage of 100 V for 1 hour, using the blotting solution (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). Following the washing of the blotted PVDF membrane with distilled water, the membrane was blocked for 3 hours in TTBS solution containing 5% Block Ace. After washing the PVDF membrane with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween20, pH 7.5), it was reacted overnight at 4°C with a primary antibody solution, rabbit polyclonal anti-megsin peptide IgG diluted in TTBS.

[0043]

Then it was detected with Amplified Alkaline Phosphatase Immuneblot Kit (BioRad). That is, it was first incubated for 1 hour with biotinylated goat anti-rabbit IgG diluted with TTBS at room temperature, then reacted with streptavidin-biotinylated alkaline phosphatase complex, which was prepared in advance by incubating streptavidin and biotinylated alkaline phosphatase for 1 hour at room temperature. The PVDF membrane was washed in TTBS, and then incubated with the substrate (nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt solution) for about 30 minutes at room temperature to visualize the antibody bound to the primary antibody. The reaction was quenched by reacting

with a sufficient amount of distilled water. The visualized bands were quantitatively analyzed using Densitograph gel documentation system (AE-6920M-05, Atto) and densitograph analyzing software (AE-6920WSE/MSE, Atto). The result (electrophoretogram) is shown in

5   Fig. 5. The measurements of the image analyzer are as follows. Dense bands were observed for IgA nephropathy patients compared to the bands of concentrated urine of normal healthy persons. It was revealed that quantitative measurement of the megsin protein is possible by quantitatively analyzing the density of these bands.

10   Measurement result of the image analyzer

Lane	Measurement result
1	Molecular weight marker
2	8.5
3	5.2
4	13.8
5	4.8
6	32.8
7	15.1

[0044]

[Example 7] Preparation of granules for detection

     The granules for detection of the present invention were

15   produced by conjugating anti-megsin peptide IgG to commercially available magnetic granules through anti-mouse IgG antibody. Dynabeads™ M-450 tosylactivated (homogenous tosyl activated paramagnetic polystyrene granules: Dynal Biotech) was used as the granules of the present invention, and a 1mM hydrochloric acid

20   solution thereof was washed once with sterile distilled water and then with 0.2 M boric acid buffer (pH9.5). Then, anti-megsin peptide IgG antibody was dissolved in 0.2 M boric acid buffer (pH 9.5) at a concentration of 150 µg/mL, and this solution was added to an equal amount of the activated granule suspension (antibody/granule=75

25   µg/15mg). Then, the mixture was incubated for 24 hours at 22°C with gentle stirring. The antibody-conjugated granules were collected with a magnet, and the supernatant was discarded leaving the granules attached to the magnet. Then the granules were washed as follows:

(1) with 5 mL 0.1 M PBS (pH7.0) for 10 minutes,

(2) with 5 mL 1 M ethanolamine-HCl (pH 9.5) for 2 hours,

(3) with 5 mL 0.05 M Tris (pH 7.5) containing 0.1 M sodium chloride, 0.1% bovine serum albumin (BSA), and 0.1% Tween20 for 12 hours, and

5 (4) with 5 mL buffer of (3) without Tween20 for 2 hours.

Thereafter, granules were collected with a magnet, supernatant was discarded, and the granules were suspended in PBS (-)/BSA containing 10% Block Ace at a concentration of about 4×10^8 granules/mL (30 mg/mL). Thus obtained IgG-conjugated granules are stable for at least 6 months

10 at 4°C.

[0045]

[Example 8] Measurement of urinary megsin protein by the magnetic granule method

Megsin protein concentration of occasional urine samples from

15 12 normal healthy persons and 24 patients with different renal diseases were measured by the magnetic granule method utilizing antibody-conjugated granules prepared in Example 7. 12 of the renal disease patients were affected with IgA nephropathy, and the other 12 were affected with minimal-change nephritic syndrome. The urinary

20 samples were kept at -80°C, and were thawed before measurement. They were centrifuged at 3,000 rpm for 5 minutes, and the supernatant thereof were used as the sample. The measurement was carried out as follows:

500,000 magnetic granules conjugated to the primary antibody

25 (rabbit polyclonal anti-megsin peptide-2 antibody) were put into a tube (1.5 mL) blocked beforehand with Block Ace (Dainippon Pharmaceutical). 500 µL of each urinary sample and equal amounts of alkaline phosphatase labeled rabbit polyclonal anti-megsin peptide-1 antibodies dialyzed with PBS (-) were mixed, and reacted for 2 hours

30 at room temperature while rotating. Then, after centrifugation at 3,000 rpm for 5 minutes, leaving the granules attached to the magnet, the supernatant was discarded, and 1mL washing solution (0.05% (w/v) Tween 20 (Wako Pure Chemical) containing PBS (-) (pH7.4) (Tween-PBS)) was added and the granules were washed while stirring. The washing

35 was repeated 4 times, and then, 100 µL substrate solution was added to react for 20 to 30 minutes with stirring. 1 M diethanolamine (Wako

Pure Chemical) buffer (pH 9.8) containing 1 mg/mL orthophenylenediamine (Wako Pure Chemical) was used as the substrate solution. Following the completion of the reaction, the reaction was quenched with 50 µL 1 N sodium hydroxide, and absorbance at 405 nm was measured. The urinary megsin protein concentration was calculated from the calibration curve. Creatinine concentration of the same urinary sample was measured as in Example 5, and the megsin protein concentration was corrected as creatinine ratio.

According to the result of the measurement, urinary megsin protein measurement values (creatinine correction value: AU) of IgA nephropathy patients were distributed in the top region compared to those of the normal healthy person. Additionally, the urinary megsin protein measurement values of IgA nephropathy patients were also higher than those of minimal-change nephritic syndrome patients. Thus, according to the present invention, it is possible not only to decide whether a person is affected by a renal disorder, but definitely identify the state of the renal disorder.

[0046]

[Effects of the Invention]

The present invention facilitates the evaluation of renal functions not by a highly invasive test like renal biopsy, but by using invasive samples like urine and blood. The realization of a simple *in vitro* evaluation of renal functions enables a quick and inexpensive test service that can be provided to many more subjects than before. There are many renal diseases, in which it is better to delay the initiation of dialysis treatment by an early detection of the diseases. Thus, the present invention not only eases the burden on the subjects, but also enables detection and observation of more patients who would need dialysis treatment in the future by providing methods for evaluating renal functions with simple examination procedures. Furthermore, a high evaluation reliability can be expected thanks to the excellent indicator, the megsin protein found in biological specimens like urine and blood, which reflects renal function.

Particularly, the immunological detection method of the megsin protein, and the kit used for the method, which are provided by the

present invention, are superior in manipulatability utilizing magnetic granule carriers. Quick and accurate measurement of the megsin protein is enabled by the present detection method. Furthermore, since the method enables the understanding of the degree of mesangial cell proliferation, the present invention makes it easy to determine the timing of when to commence or whether or not to continue steroid therapy used for treating IgA nephropathy.

[0047]

[Sequence Listing]

SEQUENCE LISTING

<110> MIYATA, Toshio
KUROKAWA, Kiyoshi
Fuso Pharmaceutical Industries, Ltd.

<120> A method of Detecting the Megsin Protein, and Use Thereof.

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<303> J. Clin. Invest.

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 65                  70                  75                  80

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His Gln Glu Arg Lys Phe Asn Leu Ser Val Ile Glu Asp Pro Ser Met

10   210   215   220

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225   230   235   240

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                245   250   255

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            260   265   270

20

Glu Val Phe Phe Pro Gln Phe Lys Ile Glu Lys Asn Tyr Glu Met Lys
        275   280   285

Gln Tyr Leu Arg Ala Leu Gly Leu Lys Asp Ile Phe Asp Glu Ser Lys

25   290   295   300

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305   310   315   320

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35

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                355   360   365

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    1   5   10   15

Cys

<210> 14
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10      15               20               25               30


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                        35               40               45
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                        50               55               60


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30      95               100              105              110


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                        115              120              125
35
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                        290                 295                 300


        tcc agg gct gat ctg tct gga att gcc tct gga ggt cgt ctc tat gta   961
        Ser Arg Ala Asp Leu Ser Gly Ile Ala Ser Gly Gly Arg Leu Tyr Val
10                  305                 310                 315


        tca aag cta atg cac aag tcc ctc ata gag gtc tca gaa gaa ggc acc  1009
        Ser Lys Leu Met His Lys Ser Leu Ile Glu Val Ser Glu Glu Gly Thr
                    320                 325                 330

15
        gag gca act gct gcc aca gaa agt aac atc gtt gaa aag cta ctt cct  1057
        Glu Ala Thr Ala Ala Thr Glu Ser Asn Ile Val Glu Lys Leu Leu Pro
        335                 340                 345                 350


20      gaa tcc acg gtg ttc aga gct gac cgc ccc ttt ctg ttt gtc att agg  1105
        Glu Ser Thr Val Phe Arg Ala Asp Arg Pro Phe Leu Phe Val Ile Arg
                        355                 360                 365


        aag aat ggc atc atc tta ttt act ggc aaa gtc tcg tgt cct          1147
25      Lys Asn Gly Ile Ile Leu Phe Thr Gly Lys Val Ser Cys Pro
                        370                 375                 380


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30      aattgtaatt ggaagtacat gg                                          1229



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Leu Ser Ile Phe Thr Ala Leu Ser Leu Ile Arg Leu Gly Ala Arg Gly
            35   40   45

Asp Cys Xaa Arg Gln Ile Asp Lys Ala Leu His Phe Ile Ser Pro Ser
        50   55   60

Arg Gln Gly Asn Ser Ser Asn Ser Gln Leu Gly Leu Gln Tyr Gln Leu
    65   70   75   80

Lys Arg Val Leu Ala Asp Ile Asn Ser Ser His Lys Asp Xaa Lys Leu
                85   90   95

Ser Ile Ala Asn Gly Val Phe Ala Glu Lys Val Phe Asp Phe His Lys
                100   105   110

Ser Tyr Met Glu Cys Ala Glu Asn Leu Tyr Asn Ala Lys Val Glu Arg
            115   120   125

Val Asp Phe Thr Asn Asp Ile Gln Glu Thr Arg Phe Lys Ile Asn Lys
        130   135   140

Trp Ile Glu Asn Glu Thr His Gly Lys Ile Lys Lys Val Leu Gly Asp
    145   150   155   160

Ser Ser Leu Ser Ser Ser Ala Val Met Val Leu Val Asn Ala Val Tyr
                165   170   175

Phe Lys Gly Lys Trp Lys Ser Ala Phe Thr Lys Ser Asp Thr Leu Ser

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                 180                    185                      190

        Cys His Phe Arg Ser Pro Ser Gly Pro Gly Lys Ala Val Asn Met Met
                   195                  200                205
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        His Gln Glu Arg Arg Phe Asn Leu Ser Thr Ile Gln Glu Pro Pro Met
                   210                  215                220

        Gln Ile Leu Glu Leu Gln Tyr His Gly Gly Ile Ser Met Tyr Ile Met
 10     225                  230                  235                240

        Leu Pro Glu Asp Asp Leu Ser Glu Ile Glu Ser Lys Leu Ser Phe Gln
                        245                  250                  255

 15     Asn Leu Met Asp Trp Thr Asn Ser Arg Lys Met Lys Ser Gln Tyr Val
                        260                  265                  270

        Asn Val Phe Leu Pro Gln Phe Lys Ile Glu Lys Asp Tyr Glu Met Arg
                   275                  280                  285
 20
        Ser His Leu Lys Ser Val Gly Leu Glu Asp Ile Phe Val Glu Ser Arg
                   290                  295                  300

        Ala Asp Leu Ser Gly Ile Ala Ser Gly Gly Arg Leu Tyr Val Ser Lys
 25     305                  310                  315                320

        Leu Met His Lys Ser Leu Ile Glu Val Ser Glu Glu Gly Thr Glu Ala
                        325                  330                  335

 30     Thr Ala Ala Thr Glu Ser Asn Ile Val Glu Lys Leu Leu Pro Glu Ser
                        340                  345                  350

        Thr Val Phe Arg Ala Asp Arg Pro Phe Leu Phe Val Ile Arg Lys Asn
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ttc ttc tct tcc ctg agc atc ttc act gcc ctg acc cta atc cgt ctg   96
Phe Phe Ser Ser Leu Ser Ile Phe Thr Ala Leu Thr Leu Ile Arg Leu
            20   25   30

ggt gct cga ggt gac tgt gca cgt cag att gac aag gca ctg cac ttt   144
Gly Ala Arg Gly Asp Cys Ala Arg Gln Ile Asp Lys Ala Leu His Phe
        35   40   45

aac ata cca tca aga caa gga aac tca tct aat aat cag cca gga ctt   192
Asn Ile Pro Ser Arg Gln Gly Asn Ser Ser Asn Asn Gln Pro Gly Leu
        50   55   60

cag tat caa ttg aaa aga gtt ctt gct gac ata aac tca tct cat aag   240
Gln Tyr Gln Leu Lys Arg Val Leu Ala Asp Ile Asn Ser Ser His Lys
 65   70   75   80

gat tat gaa ctc agc att gcc act gga gtt ttt gca gaa aaa gtc tat   288
Asp Tyr Glu Leu Ser Ile Ala Thr Gly Val Phe Ala Glu Lys Val Tyr
                    85   90   95

5

gac ttt cat aag aac tac att gag tgt gct gaa aac tta tac aat gct   336
Asp Phe His Lys Asn Tyr Ile Glu Cys Ala Glu Asn Leu Tyr Asn Ala
                100   105   110

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     Lys Val Glu Arg Val Asp Phe Thr Asn Asp Val Gln Asp Thr Arg Phe
                115   120   125

     aaa att aat aaa tgg att gaa aat gag aca cat gga aag atc aag aag   432
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            130   135   140

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20   145   150   155   160

     aac gct gtt tac ttc aaa ggc aaa tgg aaa tcg gcc ttc acc aag act   528
     Asn Ala Val Tyr Phe Lys Gly Lys Trp Lys Ser Ala Phe Thr Lys Thr
                    165   170   175
25

     gat acc ctc agt tgc cgt ttt agg tct ccc acg tgt cct gga aaa gta   576
     Asp Thr Leu Ser Cys Arg Phe Arg Ser Pro Thr Cys Pro Gly Lys Val
                180   185   190

30   gtt aat atg atg cat caa gaa cgg cgg ttc aat ttg tct acc att cag   624
     Val Asn Met Met His Gln Glu Arg Arg Phe Asn Leu Ser Thr Ile Gln
                195   200   205

     cag cca cca atg cag gtt ctt gag ctc caa tat cat ggt ggc ata agc   672
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                210   215   220

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      225                 230             235             240
  5
      ctg agt ttc cag aat ctg atg gac tgg acc aat agg agg aaa atg aaa      768
      Leu Ser Phe Gln Asn Leu Met Asp Trp Thr Asn Arg Arg Lys Met Lys
                        245             250             255

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      Ser Gln Tyr Val Asn Val Phe Leu Pro Gln Phe Lys Ile Glu Lys Asn
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      tat gaa atg acg cac cac ttg aaa tcc tta ggc ttg aaa gat atc ttt      864
 15   Tyr Glu Met Thr His His Leu Lys Ser Leu Gly Leu Lys Asp Ile Phe
                275             280             285

      gat gag tcc agt gca gat ctc tct gga att gcc tct gga ggt cgt ctc      912
      Asp Glu Ser Ser Ala Asp Leu Ser Gly Ile Ala Ser Gly Gly Arg Leu
 20       290             295             300

      tac gta tca aag cta atg cac aag tca ttc ata gag gtc tca gag gag      960
      Tyr Val Ser Lys Leu Met His Lys Ser Phe Ile Glu Val Ser Glu Glu
      305             310             315             320
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      ggc act gaa gcc act gct gcc aca gaa aat aac att gtt gaa aag cag     1008
      Gly Thr Glu Ala Thr Ala Ala Thr Glu Asn Asn Ile Val Glu Lys Gln
                        325             330             335

 30   ctt cct gag tcc aca gtg ttc aga gcc gac cgc ccc ttt ctg ttt gtc     1056
      Leu Pro Glu Ser Thr Val Phe Arg Ala Asp Arg Pro Phe Leu Phe Val
                    340             345             350

      atc aag aag aat gac atc atc tta ttt act ggc aaa gtc tct tgt cct     1104
 35   Ile Lys Lys Asn Asp Ile Ile Leu Phe Thr Gly Lys Val Ser Cys Pro
                355             360             365
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15   20   25   30

    Gly Ala Arg Gly Asp Cys Ala Arg Gln Ile Asp Lys Ala Leu His Phe
                35   40   45

20 Asn Ile Pro Ser Arg Gln Gly Asn Ser Ser Asn Asn Gln Pro Gly Leu
                50   55   60

    Gln Tyr Gln Leu Lys Arg Val Leu Ala Asp Ile Asn Ser Ser His Lys
     65   70   75   80

25
    Asp Tyr Glu Leu Ser Ile Ala Thr Gly Val Phe Ala Glu Lys Val Tyr
                     85   90   95

    Asp Phe His Lys Asn Tyr Ile Glu Cys Ala Glu Asn Leu Tyr Asn Ala
30   100   105   110

    Lys Val Glu Arg Val Asp Phe Thr Asn Asp Val Gln Asp Thr Arg Phe
                115   120   125

35 Lys Ile Asn Lys Trp Ile Glu Asn Glu Thr His Gly Lys Ile Lys Lys
                130   135   140

Val Leu Gly Asp Ser Ser Leu Ser Ser Ser Ala Val Met Val Leu Val
145   150   155   160

5   Asn Ala Val Tyr Phe Lys Gly Lys Trp Lys Ser Ala Phe Thr Lys Thr
                 165   170   175

Asp Thr Leu Ser Cys Arg Phe Arg Ser Pro Thr Cys Pro Gly Lys Val
             180   185   190

10
Val Asn Met Met His Gln Glu Arg Arg Phe Asn Leu Ser Thr Ile Gln
         195   200   205

Gln Pro Pro Met Gln Val Leu Glu Leu Gln Tyr His Gly Gly Ile Ser
15   210   215   220

Met Tyr Ile Met Leu Pro Glu Asp Gly Leu Cys Glu Ile Glu Ser Lys
225   230   235   240

20   Leu Ser Phe Gln Asn Leu Met Asp Trp Thr Asn Arg Arg Lys Met Lys
                 245   250   255

Ser Gln Tyr Val Asn Val Phe Leu Pro Gln Phe Lys Ile Glu Lys Asn
             260   265   270

25
Tyr Glu Met Thr His His Leu Lys Ser Leu Gly Leu Lys Asp Ile Phe
         275   280   285

Asp Glu Ser Ser Ala Asp Leu Ser Gly Ile Ala Ser Gly Gly Arg Leu
30   290   295   300

Tyr Val Ser Lys Leu Met His Lys Ser Phe Ile Glu Val Ser Glu Glu
305   310   315   320

35   Gly Thr Glu Ala Thr Ala Ala Thr Glu Asn Asn Ile Val Glu Lys Gln
                 325   330   335

Leu Pro Glu Ser Thr Val Phe Arg Ala Asp Arg Pro Phe Leu Phe Val
      340   345   350

5   Ile Lys Lys Asn Asp Ile Ile Leu Phe Thr Gly Lys Val Ser Cys Pro
      355   360   365

[Brief Description of the Drawings]
   [Fig. 1]

10   Fig. 1 shows the result of Western blotting using a polyclonal antibody specific to synthetic peptide-2, which has a partial amino acid sequence of megsin protein (SEQ ID NO: 12), as the antigen. Each lane represents the following:

1: MBP

15 2: MBP-megsin protein

3: His-megsin protein

4: MBP-PAI II

5: megsin protein expressed in CHO cells

6: body fluid of the silkworm infected with a recombinant virus

20 transfected with the megsin protein gene

7: body fluid of the silkworm infected with a recombinant virus without transfection by megsin protein gene

8: body fluid of the virus-uninfected silkworm

9: normal human serum

25   [Fig. 2]

   Fig. 2 shows the result of Western blotting using a polyclonal antibody specific to synthetic peptide-3, which has a partial amino acid sequence of megsin protein (SEQ ID NO: 13), as the antigen. Each lane represents the same proteins as in Fig. 1.

30   [Fig. 3]

   Fig. 3 shows the result of Western blotting using a polyclonal antibody specific to synthetic peptide-342, which has a partial amino acid sequence of megsin protein (SEQ ID NO: 15), as the antigen. Each lane represents the same proteins as in Fig. 1.

35   [Fig. 4]

   Fig. 4 is a graph showing assay results of urinary megsin

proteins by ELISA. The vertical axis shows assayed value of megsin protein (creatinine ratio: AU). Urine from normal healthy person (16 samples), from patients with IgA nephropathy (24 samples), and from patients with minimal-change nephritic syndrome (24 samples) was used

5   as specimens.

[Fig. 5]

Fig. 5 shows the assay result of Western blotting on urinary megsin protein. Each lane represents the following:

1: MBP-megsin protein

10   2 to 5: concentrated urine from normal healthy persons

6 and 7: concentrated urine from patients with IgA nephropathy

[Document Name] Drawings

[Fig. 1]

ANTI-MEGSIN PEPTIDE 2 IgG
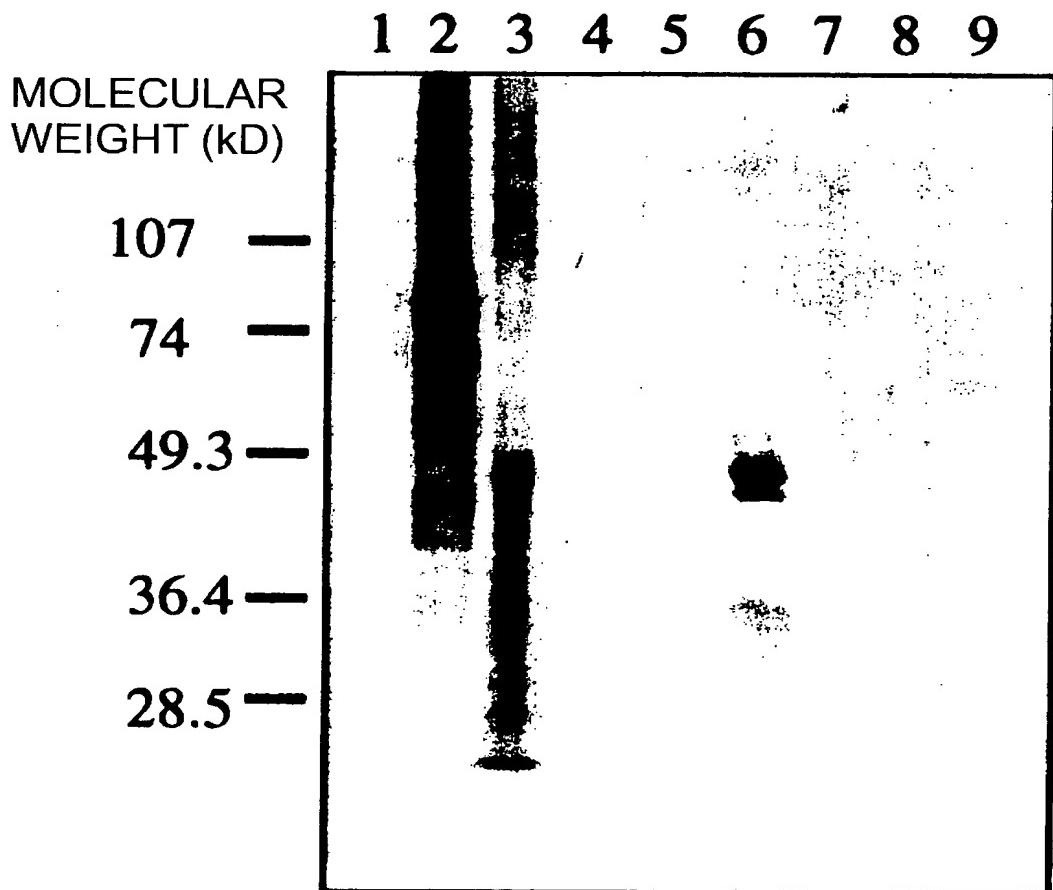
[Fig. 2]

ANTI-MEGSIN PEPTIDE 3 IgG
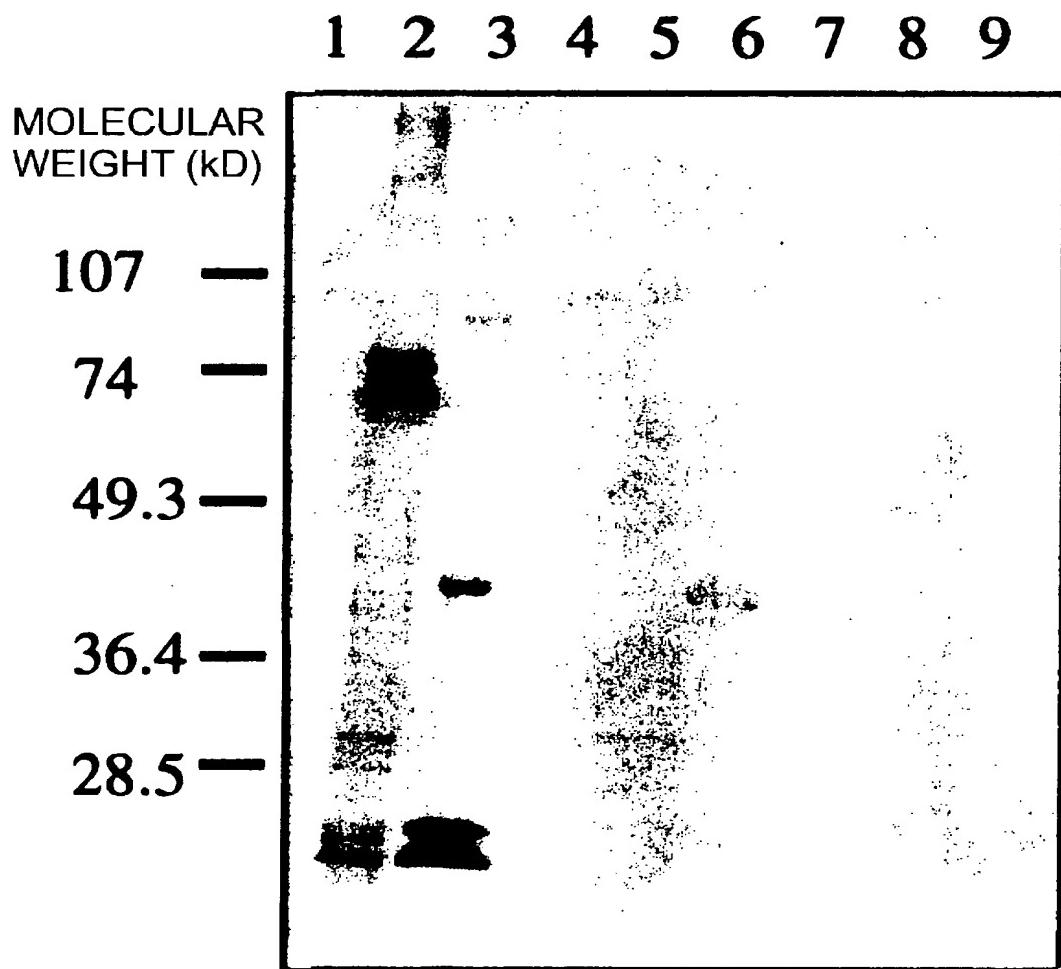
[Fig. 3]

ANTI-MEGSIN PEPTIDE 342 IgG
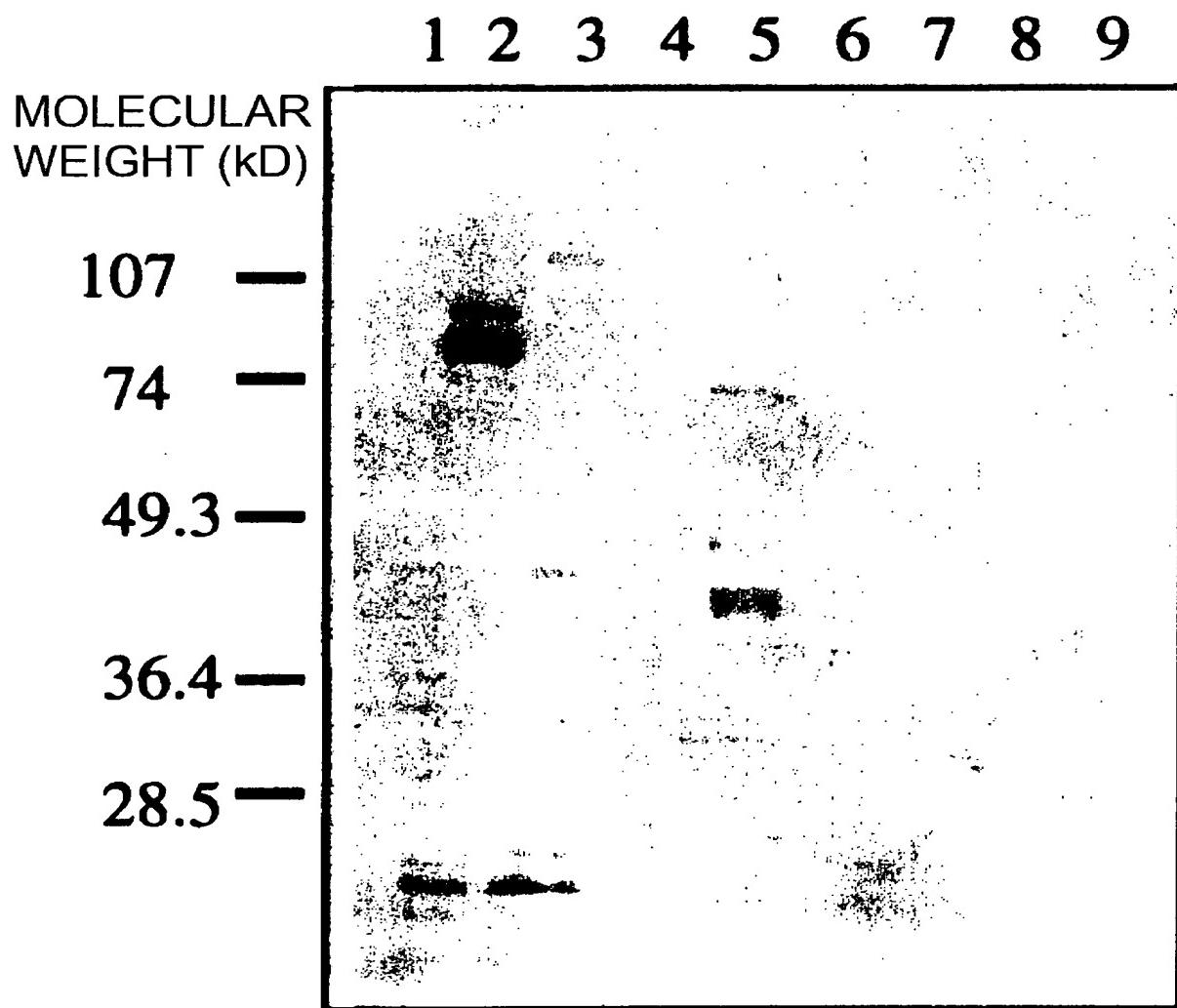
[Fig. 4]
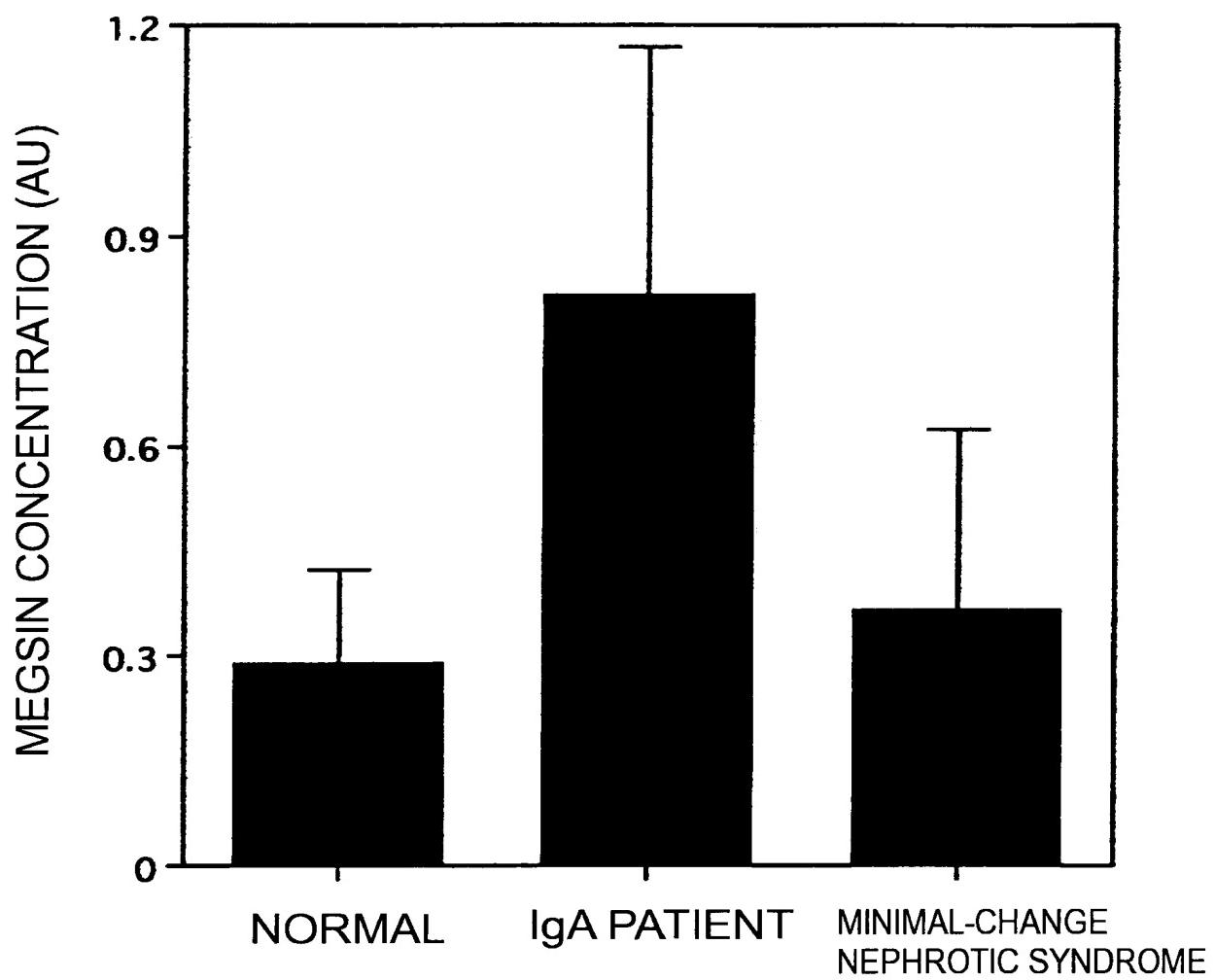
[Fig. 5]
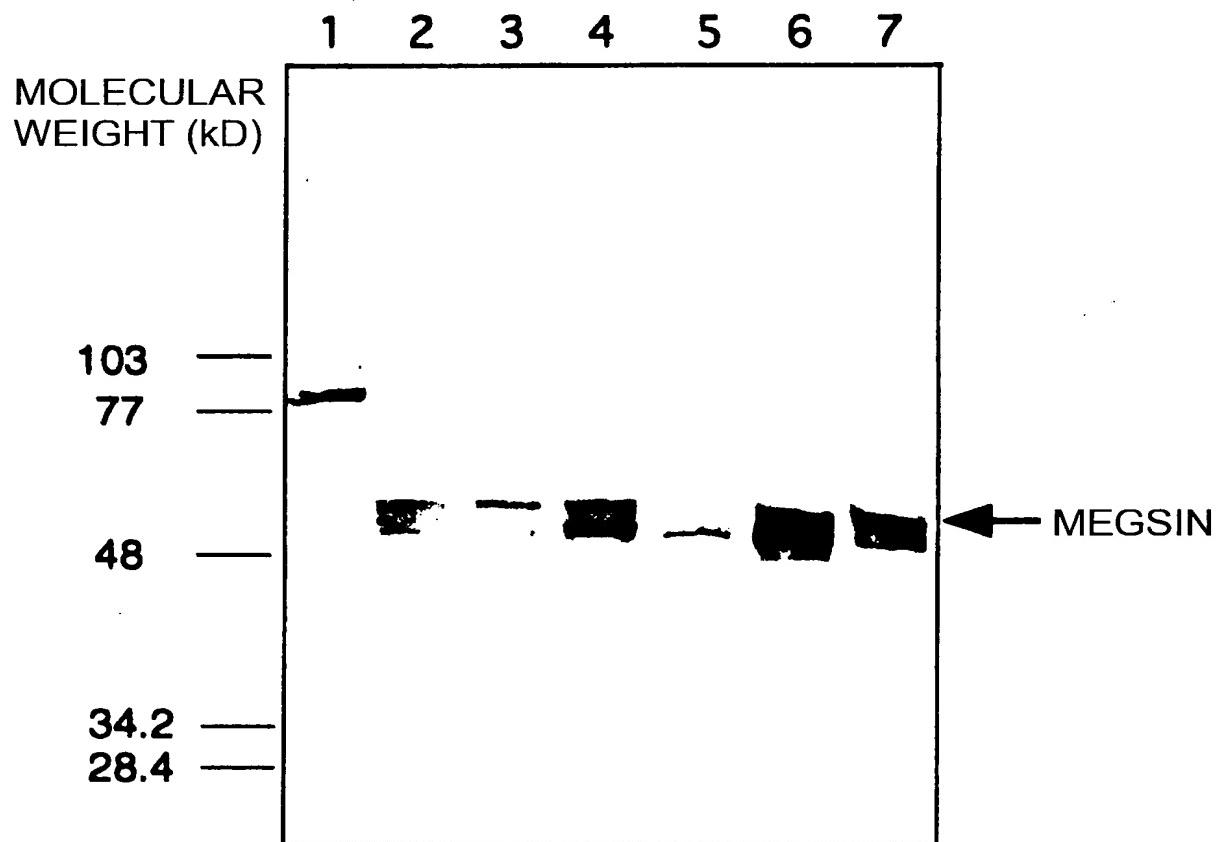
[Document Name] Abstract

[Abstract]

[Problems to Be Solved] The present invention provides a method that enables diagnosis of renal diseases and determination of their severity *in vitro*.

[Means to Solve the Problems] The present invention provides a method for evaluating renal functions by measuring megsin protein in a biological specimen. Moreover, the present invention provides immunological assay reagents, as well as kits useful for the measurement of megsin protein in the biological specimen.

[Selected Drawings] None